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RESEARCH PAPER

Effects of *in vitro* subculture on the physiological characteristics of adventitious root formation in microshoots of *Castanea mollissima* cv. 'yanshanhong'

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Abstract: Microshoots of *Castanea mollissima* cv. 'yanshanhong' *in vitro* acquired an enhanced rooting capability with increasing numbers of subculture. In this study, we investigated the effect of successive subculture on adventitious root formation *in vitro* by the determination of the endogenous hormone level and the enzyme activity. The levels of indoleacetic acid (IAA), abscisic acid (ABA), cytokinins (CTK) and gibberellic acid (GA_3) were determined by high performance liquid chromatography (HPLC), and the activities of indoleacetic acid oxidase (IAAO), peroxidase oxidase (POD), and polyphenol oxidase (PPO) were measured by ultraviolet-spectrophotometer assay after the induction of rooting at 2nd, 4th, 6th and 8th subculture. The relationships between physiological characteristics and subculture numbers or rooting rate were as follows: The levels of endogenous IAA in microshoots gradually increased, and endogenous levels of ABA, CTK and GA_3 in microshoots decreased slightly after serial subcultures. The level of IAA was highly correlated with subculture numbers and rooting rates. The ratios of IAA/ABA and IAA/CTK both acutely raised with increasing rooting rate during successive subcultures and had high correlations with rooting rate. The activity of IAAO and POD are significantly negatively related with subculture numbers, and the activity of PPO increased after subcultures.

Keywords: adventitious root formation; subculture; endogenous hormone; enzyme

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Introduction

Chinese chestnut (*Castanea mollissima* BL) has many fine and rare varieties. It plays a very important role in world chestnut development because of improved production of nuts and high resistance to chestnut blight (Grave 1950; Huang 1998). Regarded as the best way to rapidly propagate, tissue culture of *C. mollissima* is the significant part of breeding fine varieties. Whereas, low rooting rate is always the limiting factor of consistently successful tissue culture of chestnut on a commercial scale (Xing 1997).

Many researches indicate that difference of subculture numbers in culture material has a direct effect on the rooting capacity of microshoots. For the difficult-to-root apple (*Malus pumila* Mill cv. Fuji, Starkrimson) and cultivar 'Jonathan' (*Malus*× *domestica* Borkh.), proliferating axillary shoots acquired an enhanced rooting ability with increasing the number of subcultures *in vitro* (Chang et al. 1991; Noiton et al. 1992). Precocious walnut (*Juglans regia* L.) shoots have a high rooting rate by induction *in vitro* after they have been subcultured for 4 years (Pei et al. 2002). Wang and Guo (2007) found that the microshoots of *Castanea mollissima* cv. 'yanshanhong' did not achieve the significantly high rooting rate until the 8th generation.

Adventitious root formation and development go through three phases (induction, initiation and expression), (Jarvis 1986). Kinds of endogenous hormones are involved, including indoleacetic acid (IAA), abscisic acid (ABA), cytokinin (CTK) and gibberellins (GA). These phytohormones' effection are variously complicated and correlative in different species (Pan and Tian 1999; Wang et al. 2005). Meanwhile, the activities of three varieties of enzymes- indoleacetic acid oxidase (IAAO), peroxidase oxidase (POD) and polyphenol oxidase (PPO) are closely related to adventitious root formation (Xiao et al. 2002; Huang et al. 2002).

As above, the repeated subculture is the efficient way for forming adventitious root in many species of woody-plant; and the relevant mechanisms of adventitious root formation are in-

volving hormone level and enzyme activity. However, few studies have revealed the mechanisms of successive subcultures of microshoots *in vitro* by analyzing hormone level and enzyme activity in rooting period of different generations. The present study aimed to discover the mechanisms and the relationships between the change of these physiological characteristics of microshoots during the adventitious root formation and the increasing subculture number and increasing rooting ability on the ground of Wang and Guo's (2007) research.

Materials and methods

Culture initiation

Plant seeds of *Castanea mollissima cv*. 'Yanshanhong' were supplied by the experimental station in Huairou District in Beijing. The seedlings were grown in the glasshouse under natural illumination. Nodes with 3−4 buds were removed from shoot tips of 2−3-month-old seedlings as explants. Single node explants were surface-sterilized and transferred onto Murashige and Skoog (MS)($1/2NO_3^-$) medium supplemented with sucrose of 30 $g \cdot L^{-1}$, agar of 7 $g \cdot L^{-1}$, 6- benzyladenine (6-BA) of 0.5 $mg \cdot L^{-1}$ for inducing shoot growth from axillary buds, and 100-$mg \cdot L^{-1}$ β-mercaptoethanol (PVP) for preventing browning. The pH was adjusted to 5.8−6.0 with NaOH before autoclaving at 121ºC for 20 min.

Shoot proliferation and rooting

After four weeks on initial medium, new axillary shoots were excised from the nodes and transferred onto MS ($1/2NO_3^-$) medium supplemented with 0.5-$mg \cdot L^{-1}$ 6-BA (proliferation medium). During the proliferation phase, microshoots were subcultured every four weeks. The initial nodal explants were marked as culture 1 and the shoots resulting from the growth of these buds were called subculture 2. The proliferating shoots from subculture 2 were designated as subculture 3 and so on.

A two-step procedure to induct adventitious root formation was adopted. In the first step, individual microshoots were excised and transferred to half-strength MS ($1/2NO_3^-$) supplemented with 3.0-$mg \cdot L^{-1}$ 3-indole-butyric acid (IBA) for root induction and cultured in dark for five days. After then, these shoots were transferred to semi-MS ($1/2NO_3^-$) auxin-free (Wang, 2007). All cultures were incubated in a growth chamber under the condition of (24±1) ºC and 16-hour light and 8-hour dark.

Collection and determination

Microshoots were collected from each of the 2^{nd}, 4^{th} , 6^{th} , 8^{th} subculture roots inducted by using IBA. The shoots' bases were separately excised and immediately frozen in liquid nitrogen, and preserved in hypothermic freezer. Analyses were performed in triplicate and each sample consisted of 10 microshoots.

Analysis of endogenous hormone

Around 1.0-g freeze-dried material with few sodium diethyldityiocarbamate was grinded in 80% iced methanol to homogenate. After washing up by iced methanol, the homogenate was transferred into small beaker with tin foil cover and conserve in deep-freeze at 4ºC in darkness for 15 h. The extract was filtered through Buchner funnel, after added one or two drops ammonia. It was concentrated to aqueous phase on rotary evaporator at 36ºC, and then freezed/thawed three times. The clear liquid in the sample was collected by centrifuge (8000 rpm, 25 min, 4ºC). The sample 1 for analyzing GA_3, IAA and ABA was adjusted at pH 2.5−3.0. The sample 2 for analyzing CTK was adjusted at pH 7.5−8.0. The sample 1 was extracted by equal volume of ethyl acetate three times and combined organic phase each time. The sample 2 was extracted by phosphate buffer saturated buthanol (pH=8) three times and combined organic phase each time. Sample 1 and sample 2 were concentrated on rotary evaporator at 35ºC–40 ºC and 60 ºC. The concentrated sample was dissolved by mobile phase and constant volume at 1 mL for HPLC.

After purification, the methanolic extracts were injected in a Water HPLC system (Agilent 1100 series, chromatogram column, 250mm×4.0mm, 5.0μm). The flow rate was 1 $mL \cdot min^{-1}$. The mobile phase was 3% methanol and 0.1N ethanoic acid for sample 1, and 3% methanol and 97% water (pH=7) for sample 2. The diode array detection wavelengths were 210 nm, 254 nm and 280 nm for sample 1 to detect the concentration of GA_3, IAA and ABA; CTK concentration was measured at wavelength 254 nm with sample 2.

$$C=(S_1\times N_0\times V_0)/(S_0\times V_1) \quad (1)$$

where, C is the concentration of measuring sample; S_1 is the peak area of measuring sample, S_0 the peak area of standard sample; V_0 the injection volume of standard sample (μL), V_1 the injection volume of measuring sample (μL), and N_0 is the concentration of standard sample (19.13$\mu g \cdot mL^{-1}$ of ABA, 51.13$\mu g \cdot mL^{-1}$ of IAA, 994.78$\mu g \cdot mL^{-1}$ of GA_3, 500$\mu g \cdot mL^{-1}$ of CTK).

Analysis of enzyme

The enzyme active of sample was measured with the method described by Zhang (1998). Briefly, the sample of microshoots (1 g) was grinded to homogenate with 2-mL cold phosphate buffer and a few quartz sands into mortar in ice bath. After the homogenate was diluted to 5 mL, the clear liquid in the sample was collected by centrifuge (8500 rpm, 20 min, 4ºC) and constant volume again.

Analysis of IAAO: 0.5-mL enzyme solution was added into reaction solution (1 mL of 1×10^{-3} $mol \cdot L^{-1}MnCl_2$, 1 mL of 1×10^{-3} $mol \cdot L^{-1}$ 2,4-Dichlorophenol, 1 mL of 1×10^{-3} $mol \cdot L^{-1}$ IAA) and mixed in tube 1. Phosphate buffer was added into the same reaction system in tube 2. In tube 3 within distilled water was control. The 1 mL of each mixed solution was separately added 2-mL

$FeCl_3$-perchloric acid reagent, shaken up and conserved in thermostat (35ºC) for 30 min in darkness. The absorbance value was determined at 530 nm.

Analysis of POD: After 0.1-mL enzyme solution was added into reaction solution (2.9 mL of 0.05 mol/L phosphate buffer, 1.0 mL of 2% H_2O_2, 1.0 mL of 0.05 mol/L guaiacol) in tube, it was put in water bath (30 ºC) for 3 min immediately, then added 1.0 mL of 2% H_2O_2 in water bath for 2 min and transferred the solution into colorimetric ware. The absorbance value was determined at 470 nm.

Analysis of PPO: After 3-mL phosphate buffer (pH=6.0) and 0.1-mL enzyme solution were reacting exactly 1 min in tube, it was added by 1-mL pyrocatechol (0.08 mol·L^{-1}) immediately and shaken up. The absorbance value was determined at 535 nm.

Statistical analysis

The data were analyzed by variance analysis, LSD multiple comparative test at 95% and 99% level, and one sample T test. The protracted curves were protracted using EXCEL and SPSS statistics software.

Results

Effects of subculture on endogenous hormone level

The results of variance analysis showed that the level of IAA and GA_3 had significant differences between different subcultures (Table 1). The level of IAA was improved with increasing the number of subcultures in microshoots pre-treated with IBA to induce adventitious root formation. Microshoots in subculture 6 had a significant higher IAA content than former subculture. IAA content in subculture 8 kept increasing and was 4 times higher than that in subculture 2. The correlation between IAA content and rooting rate was 0.963. And the correlation between IAA content and subculture number was 0.968. They were both significant at the 0.05 level (2-tailed). There was no variation in the endogenous level of ABA through the successive subcultures. The endogenous GA_3 and CTK concentration presented a decreasing trend with the increase of the number of subcultures. The content of GA_3 of microshoots in subculture 6 was significantly lower than that of former subculture. The content of CTK had a transient increase in subculture 4, but it decreased from subculture 4 to subculture 8 (Fig. 1). Compared to single endogenous hormone level, the ratios between two levels of endogenous hormone show more regular changing tendency through serial subcultures (Fig. 2). The ratio between IAA and ABA content (IAA/ABA) in root formation of microshoots reached 4 at subculture 8, which is twice higher than the ratio achieved at subculture 6. Statistically significant correlation (0.986, 0.05 level, 2-tailed) was obtained between the IAA/ABA and rooting rate. An acute change was also observed in ratios of IAA to CTK from subculture 4 to subculture 8, which increased more than 10 times in this phase. The correlation (0.991) between IAA/CTK and rooting rate was significant at the 0.01 level (2-tailed).

Table 1. Effects of subculture in vitro on the physiological characteristics of adventitious root formation in microshoots of *Castanea mollissima* cv. 'yanshanhong'

Subculture number	Rooting rate (%)*	Number of roots*	IAA content (μg·g^{-1}FW)	ABA content (μg·g^{-1}FW)	GA_3 content (μg·g^{-1}FW)	CTK content (μg·g^{-1}FW)
2	0	0	0.623±0.095a	0.775±0.125a	1.162±0.237a	0.023±0.010ab
4	0	0	0.768±0.111a	0.907±0.055a	0.983±0.229a	0.032±0.007a
6	23.3	2	1.670±0.173b	0.705±0.191a	0.161±0.019b	0.022±0.003ab
8	73.3	5	2.397±0.276c	0.590±0.238a	0.095±0.018b	0.008±0.004b
Subculture number	IAA /ABA	IAA /CTK	IAAO activity (μg·g^{-1}·h^{-1}FW)	POD activity (mg^{-1}·min^{-1}·$protein^{-1}$)	PPOactivity (mg^{-1}·min^{-1}·$protein^{-1}$)	
2	0.804	27.655	138.4±32.5a	1247.2±91.4a	435.4±112.2a	
4	0.846	24.344	98.8±19.1ab	634.0±31.0a	1066.1±96.2b	
6	2.370	77.386	73.8±10.1b	511.0±17.1b	971.3±121.8b	
8	4.062	295.139	61.8±6.0b	71.5±6.9c	972.5±101.9b	

Notes: * The data of rooting rate and root number were reported previously by Wang and Guo(2007). Values followed by same letters are not significantly different at the 0.05 level.

Effects of the number of subcultures on enzyme activities

The variance analysis showed that the activities of POD and PPO had significant differences between different subcultures (Table 1). After repeated subculture, the activity of IAAO and POD in microshoots decreased during the adventitious root formation treated by IBA (Fig. 3), and their correlations with subculture numbers were -0.972 and -0.971, respectively, with significant at 0.05 level (2-tailed). The IAAO activity of tissue at subculture 6 was much lower than that at subculture 2. The change of POD activity was dramatic from subculture 2 to subculture 8 (Fig. 3), and ranged from 1247.2 to 71.5 U·mg^{-1}·min^{-1}·$protein^{-1}$. PPO activity in microshoots increased from subculture 2 to subculture 4, and then it stayed stable.

Discussion

Plant materials restore their juvenile traits as rooting capacity with increasing the number of subcultures *in vitro* in many species (Chang et al. 1991; Noiton et al. 1992; Arnaud et al. 1993). A progressive increase in rooting capacity of chestnut microshoots *in vitro* occurred only at subculture 8 (Feijó and Pais 1990; Wang and Guo 2007). Rejuvenation of mature chestnut could also caused by grafting alone or combining with BA sprays treatment (Sánchez et al. 1997), or the repeated the numbers of subcultures on medium containing a low cytokinin concentration (Giovannelli and Giannini 2000). The accumulation of cytokinin 6-BA and the induction of IBA influenced adventitious root formation. Cytokinins would act as a rejuvenating agent for mature trees (Kelly 1988). The improved rootability from long-term subcultures may result from increasingly sensitivity to the inductive treatment of target tissue (James 1983). IBA application promotes root formation but is not responsible for it (Noiton 1988).

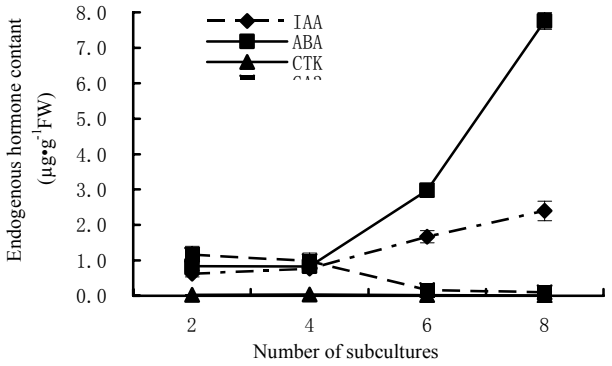

Fig. 1 Effects of subculture times on change of endogenous hormone level during adventitious root formation

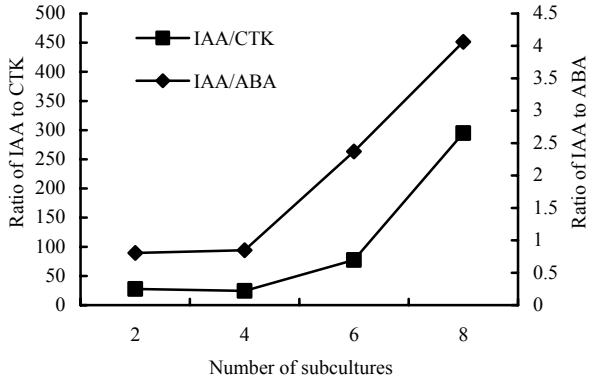

Fig. 2 Effects of subculture times on changes of IAA/ABA and IAA/CTK during adventitious root formation

High level of IAA was the key factor to induce root primordium growth (Wang et al. 2005) because cell dedifferentiation and division needed high level of endogenous auxin to induce root primordial when adventitious roots formed in the initial period (Jarvis 1986). In the present work, the effect of free IAA level is noticeable for rooting rate. We suggest that endogenous IAA is an important promotive factor of rooting and it could accumulate after subcultures. The accumulation of endogenous IAA driven by exogenous IBA is the signal responsible for root induction (Gonçalves et al. 2008). However, the reverse results appeared in some researches. The endogenous level of free IAA in difficult-to-root microshoots was similar with or higher than that in easy-to-root material (Noiton 1992; Ballester et al. 1999). Therefore, high level of endogenous IAA in materials was not the guarantee of high rooting capacity, which was also associated with the genotype of mother plants, the sensitivity of IAA receptor in microshoots and synergism with other hormones. The changes of IAA level after 8th subculture should deeply explain the relationship between IAA level and rooting capacity in chestnut microshoots in further study.

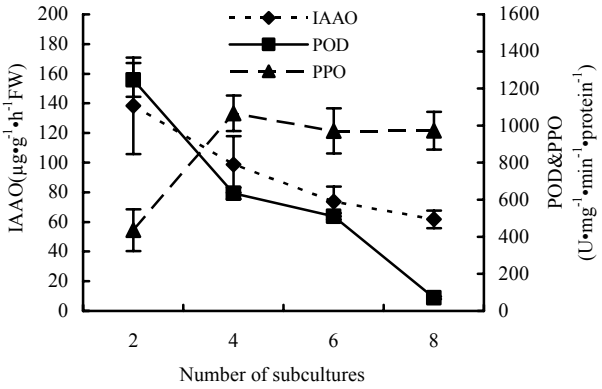

Fig. 3 Effects of subculture times on changes of enzyme activity during adventitious root formation

High ABA level was considered as the inhibition of root formation by most researches (Fouret et al. 1986; Noiton 1992). A high level of ABA was found in difficult-to-root materials whereas ABA remained constant at a low level in easy-to-root ones of 'Jonathan' apple during the 48 h after IBA treatment (Noiton 1988). However, there was no variation in the ABA level of chestnut microshoots *in vitro* throughout the subcultures in this case. The effect of endogenous ABA level on rooting ability of chestnut microshoots still couldn't be ignored because the ratio of IAA and ABA is highly correlated with rooting ability. The ratio of IAA/ABA in chestnut microshoots increased with the increasing the number of subcultures. The relationship between a high IAA/ABA ratio and an enhanced rooting ability of plant materials *in vitro* was reported in other species (Fouret et al. 1986; Noiton 1992).

CTK promoted enlargment growth of cells in cotyledons or leaves; however, it suppressed elongation growth of cells in shoots and roots (Wu 2004). Tang et al. (1996) pointed out that the low level of CTK was necessary to form root primordium, but high level of CTK would strongly suppress the root formation. In this study, endogenous CTK steadily kept at a low level during successive subcultures. Accumulation of CTK wasn't observed after serial subcultures, which might inhibit root formation (Xiao et al. 2001). The ratio of IAA to CTK has higher cor-

relation with rooting ability than the ratio of IAA to ABA. Single hormone levels were influenced by many factors and changed irregular, but their ratios were more representative. The ratios of IAA/ABA and IAA/CTK represent both positive and negative factors of endogenous hormone level, which could better explain the physiology changes of adventitious root formation on the view of balance. Further researches should explore whether the ratios could be used as a physiology indicator of adventitious root formation or not, and the standards on different species and genotypes.

The IAAO and POD played a crucial role in auxin metabolism, because the function of two enzymes was to regulate the growth of the plant by adjust the hormonal level of IAA (Yuan et al. 2008). The results show that the activities of IAAO and POD changed similarly, and had the opposite course of IAA level. The correlations between the two enzymes and IAA were -0.893 and -0.889. With the increasing the number of subcultures, the activities of the two enzymes were declined and negatively significantly correlated with the number of subcultures. According to published results, IAAO and POD were interrelated in time domain and in space during plant growth and development process and the independence of the two enzymes hadn't been proved (Yuan et al. 2008). IAAO regulates the endogenous IAA concentration and affects root formation by IAA catabolism (Dalet and Cornu 1988; Tohit 2000). The physiology function of POD is complicated. On one hand, high activity of alkaline POD activated and effected IAA metabolism to induct callus and root primordial formation (Fekete et al. 2002; Huang et al. 2002; McDonald and Wynne 2003). On the other hand, low activity of POD after root primordium formation was propitious to rooting because it's catabolism on IAA restricted root emergence and growth. Tohit (2000) suggested that POD activity played a role in cell division but had no apparent correlation with rooting ability or rooting percentage. The fluctuation of POD during the adventitious root formation should be further researched; however, the decline activity of POD in chestnut microshoots during subcultures was evidently and low average activity of POD was beneficial to rooting ability.

PPO played a role in polyphenol synthesis and inhibited lignin biosynthesis, consequently affecting cell division, cell differentiation and root primordium (Huystee and Cairns 1982; Vaughn and Duke 1984; Hayrullah et al. 2003). PPO enzyme mainly effected rooting by catalyzing 'IAA-phenol acid compound'. Additive IBA provoked the cells activity in cambium, which produced a large quantity of IAA. The increased IAA level leaded to PPO enzyme activity changes, thus inducted callus dedifferentiation (Huang et al. 2002). The change of PPO activity during the adventitious root formation may affect this process, but the difference between subcultures was not significant, and the average activity doesn't affect rooting ability.

The deficiency of this study lies on the experimental materials, which were from 2-3-month seedlings instead of mature trees with good characteres. Seedling materials can not completely represent the mature ones because of the difference of explants condition. However, for figuring out the physiology mechanism of adventitious root formation of *Castanea mollissima* in comparative short time, this experiment should be the guide of further research.

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